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# Water Networks Contribute to Enthalpy/Entropy Compensation in Protein-Ligand Binding

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## Abstract

The mechanism (or mechanisms) of enthalpy-entropy ( $H/S$ ) compensation in protein-ligand binding remains controversial, and there are still no predictive models (theoretical or experimental) in which hypotheses of ligand binding can be readily tested. Here we describe a particularly well-defined system of protein and ligands—human carbonic anhydrase (HCA) and a series of benzothiazole sulfonamide ligands with different patterns of fluorination—that we use to define enthalpy/entropy ( $H/S$ ) compensation in this system thermodynamically and structurally. The binding affinities of these ligands (with the exception of one ligand, in which the deviation is understood) to HCA are, despite differences in fluorination pattern, indistinguishable; they nonetheless reflect significant and compensating changes in enthalpy and entropy of binding. Analysis reveals that differences in the structure and thermodynamic properties of water surrounding the bound ligands are an important contributor to the observed  $H/S$  compensation. These results support the hypothesis that the molecules of water filling the active site of a protein, and surrounding the ligand, are as important as the contact interactions between the protein and the ligand for biomolecular recognition, and determining the thermodynamics of binding.

## Introduction

The hydrophobic effect—the tendency of nonpolar molecules or parts of molecules to aggregate in aqueous media—is central to biomolecular recognition. It now seems that there is no *single* “hydrophobic effect”<sup>1-4</sup> that adequately describes the partitioning of a small apolar ligand between both i) an aqueous phase and a non-polar organic phase (e.g., buffer and octanol), and ii) bulk aqueous buffer and the active site of a protein (i.e., biomolecular recognition). While the molecular-level mechanisms of hydrophobic effects in biomolecular recognition remain a subject of substantial controversy, it is clear that the water molecules surrounding the apolar ligand and filling the active site of the protein are an important part of these mechanisms.<sup>1-10</sup> Clarifying the role of water in the hydrophobic effect in protein-ligand binding would be an important contribution to understanding the fundamental, mechanistic basis of molecular recognition. Resolving this mechanism would, however, still leave a (presumably) related phenomena unresolved: so-called, enthalpy-entropy compensation (*H/S* compensation).

*H/S* compensation is often encountered in the putative design of tight-binding, low-molecular-weight ligands for a protein.<sup>11,12</sup> Changes in the structure of the ligand often lead to opposite and compensating changes in the enthalpy and entropy of binding, but result in surprisingly small changes in the free energy of binding. The molecular-level mechanism of *H/S* compensation in protein-ligand binding – and even its existence as a phenomenon<sup>12</sup> – remains a subject of substantial controversy—even at a conceptual level—despite qualitative rationalizations based upon: i) an unfavorable entropy of binding caused by conformational restrictions of the ligand upon binding;<sup>13-15</sup> ii) small conformational changes throughout the protein upon ligand binding;<sup>16,17</sup> iii) reorganization of solvent molecules within the active site of a protein after ligand binding.<sup>18-20</sup>

Human carbonic anhydrase II (HCA, EC 4.2.1.1) is an excellent model system with which to study *H/S* compensation because it allows us to answer the question: “Do changes in the structure of the networks of hydrogen bonded waters – that result upon ligand binding – determine *H/S* compensation?”. Thiazole-based sulfonamide ligands bind to HCA with an enthalpy-dominated hydrophobic effect,<sup>2,4</sup> and are not compatible with an entropy-dominated hydrophobic effect proposed by Kauzmann and Tanford.<sup>21,22</sup> or the *H/S* compensation proposed by Dunitz, Williams, and others.<sup>13-15</sup> HCA is conformationally rigid and undergoes minimal (< 1 Å) conformational changes upon binding of most arylsulfonamide ligands<sup>23</sup>—and, more importantly for this study thiazole-based sulfonamide ligands<sup>2,4</sup>—and allows us to focus solely on rearrangements of solvent within the active site of the protein, and not on contributions caused by conformational changes in the protein.

We have measured changes in the thermodynamics of binding of a series of heteroarylsulfonamide ligands to HCA with isothermal titration calorimetry (ITC) in which a benzo-, fluorobenzo-, or tetrahydrobenzo-group was added to one edge of the ligand (Figure 1A).<sup>2,4</sup> Remarkably, the free energies of binding ( $\Delta G^{\circ}_{\text{bind}}$ ) of benzothiazole sulfonamide (**H<sub>4</sub>BTA**) and perfluorobenzothiazole sulfonamide (**F<sub>4</sub>BTA**) to HCA are indistinguishable,<sup>2</sup> and suggests that the hydrophobic effects in this protein-ligand system are insensitive to this substantial change in the chemical composition and electronic structure of the ligand. While the values of  $\Delta G^{\circ}_{\text{bind}}$  of **H<sub>4</sub>BTA** are unchanged upon fluorination, we do observe significant and compensating changes in the enthalpy ( $\Delta H^{\circ}_{\text{bind}}$ ) and entropy ( $-T\Delta S^{\circ}_{\text{bind}}$ ) of binding,<sup>2</sup> indicating that the underlying mechanism of molecular recognition may be different even though the total free energy of binding is the same.

ITC thermograms of protein-ligand binding contain three values—the binding affinity of the ligand, the  $\Delta H^{\circ}_{\text{bind}}$ , and the stoichiometry of binding—that are obtained from a non-linear fit of the data. Uncertainty in the concentration of the ligand is the main source of measurement error in ITC, and leads to errors in  $K_a$  and  $\Delta H^{\circ}_{\text{bind}}$ —as well as  $\Delta G^{\circ}_{\text{bind}}$  and  $-T\Delta S^{\circ}_{\text{bind}}$ , which are calculated from these values.<sup>12,24</sup> In order to reduce artifacts in our ITC measurements that could lead to perceived *H/S* compensation we: i) measured the binding of a standard sulfonamide (methazolamide), whose concentration was determined accurately with NMR standards, to HCA and obtained the concentration of active protein; ii) prepared stock solutions of each ligand, and used these stocks solutions for each experiment to eliminate changes in the concentration of the ligand between experiments; iii) compared the binding stoichiometry of each ligand with the methazolamide standard to obtain an accurate concentration of each ligand; iv) accounted for the uncertainties associated with the nonlinear fits used to analyze the thermograms ( $\Delta H^{\circ}_{\text{bind}}$  less than ~10%), and compared the average value of ( $n = 7$  runs) each ligand with a Student's t-test with a 95% confidence interval.

In this paper we wished to determine if selectively replacing the hydrogen atoms of the benzothiazole moiety with fluorine atoms would change the network of waters in the active site of HCA, and result in an *H/S* compensation similar to that observed between **H<sub>4</sub>BTA** and **F<sub>4</sub>BTA**. Selective replacement of the C-H bonds of the benzo-group with C-F bonds allow us to study the binding of a set of ligands that are similar in size, but have entirely different group dipole moments and therefore different interactions with the networks of water in the active site.

## Results and Discussion

*Fluorination of the benzothiazole ligand changes its electronic structure, but leaves the size of the ligand and its binding geometry relatively unchanged.* Replacing all of the C – H bonds of the benzo-extension of **H<sub>4</sub>BTA** with C – F bonds results in a decrease of the average dipole moment, but does not result in large changes in: i) the pK<sub>a</sub> of the ligand (a decrease of 0.3 units); ii) the solvent accessible surface area of the ligand in crystal structures of the HCA: ligand complex (an increase of 34 Å<sup>2</sup>); iii) the binding geometry of the ligands (a shift of 0.7 Å in the binding pocket toward the hydrophobic wall).<sup>2</sup>

We compared the binding geometry of six of the partially fluorinated ligands (**4-F<sub>1</sub>BTA**, **7-F<sub>1</sub>BTA**, **5,6-F<sub>2</sub>BTA**, **6,7-F<sub>2</sub>BTA**, **4,6-F<sub>2</sub>BTA**, and **4,7-F<sub>2</sub>BTA**) to HCA with previously solved structures of HCA complexed with **H<sub>4</sub>BTA**<sup>4</sup> and **F<sub>4</sub>BTA**<sup>2</sup>. Each of the high-resolution crystal structures (Figure 2, resolutions ranging from 1.25–1.50 Å) show that a fluorine atom in the 4-position causes the ligands to: i) bind to HCA with the same geometry as **F<sub>4</sub>BTA**; ii) rotate 180° around the molecular axis of the sulfonamide–benzothiazole bond when there is not a fluorine in the 7-position. We attribute these changes in the position of the partially fluorinated ligands to a repulsive interaction between the fluorine atom of the ligand and Thr 200 (Figure 1C).

Surprisingly, the position of the amino acids lining the active site of HCA are not affected by the position or orientation of the ligand: the average root-mean square deviation (RMSD) of the heavy atoms of the amino acids lining the active site of all of the partially-fluorinated ligand with **H<sub>4</sub>BTA** and **F<sub>4</sub>BTA** is 0.132 Å and 0.112 Å, respectively.

These crystal structures suggest that the interactions between the benzothiazole ligands and the binding pocket of HCA are mediated by the molecules of water in the active site, and not through the traditional lock-and-key model of direct interactions between protein and ligand.

*The binding affinity of benzothiazole is relatively unaffected by fluorination, and in certain cases is the result of compensating values of enthalpy and entropy of binding.* We measured the enthalpies of binding ( $\Delta H_{\text{bind}}^{\circ}$ ) and the association constants ( $K_a$ ) for the series of the partially fluorinated ligands in Figure 1A with isothermal titration calorimetry (ITC), and estimated the free energies ( $\Delta G_{\text{bind}}^{\circ}$ ) and entropies ( $-T\Delta S_{\text{bind}}^{\circ}$ ) of binding. Figure 3 plots the  $pK_a$ -corrected values of  $\Delta J_{\text{bind}}^{\circ}$  for each ligand (where  $\Delta J = \Delta G$ ,  $\Delta H$ , or  $-T\Delta S$ ); these values represent the binding of the sulfonamide anion to HCA (details in the SI).<sup>4,25</sup> We classified the ligands into three categories: i) ligands in which  $\Delta H_{\text{bind}}^{\circ}$  and  $-T\Delta S_{\text{bind}}^{\circ}$  are unchanged, and result in an unchanged binding affinity (**7-F<sub>1</sub>BTA**, **5,6-F<sub>2</sub>BTA**, **4,7-F<sub>2</sub>BTA**, and **5,6,7-F<sub>3</sub>BTA**); ii) ligands in which  $-T\Delta S_{\text{bind}}^{\circ}$  is significantly different, and results in an increase in binding affinity (**6,7-F<sub>2</sub>BTA**); iii) ligands in which  $\Delta H_{\text{bind}}^{\circ}$  and  $-T\Delta S_{\text{bind}}^{\circ}$  are significantly different, but compensate and result in an unchanged binding affinity (**4-F<sub>1</sub>BTA** and **4,6-F<sub>2</sub>BTA**). A change is considered significant if the values of  $\Delta J_{\text{bind}}^{\circ}$  are statistically distinguishable from **H<sub>4</sub>BTA** at  $p < 0.05$  (by Student's t-test).

*Molecular dynamics simulations support the role of water in H/S compensation.* Our previous studies of HCA-arylsulfonamide ligand complexes support the hypothesis that the network of waters within the active site of HCA is an integral component in enthalpically driven hydrophobic effects. Structural and thermodynamic data of the partially fluorinated ligands binding to HCA suggest that the network of hydrogen-bonded water molecules in the active site of a protein-ligand complex could be responsible for changes in the  $\Delta H_{\text{bind}}^{\circ}$  and  $-T\Delta S_{\text{bind}}^{\circ}$ , but there are currently no experiments to probe the thermodynamic characteristics of individual water molecules directly in the active site of a protein. X-ray and neutron-diffraction data provide information about some of the so-called “fixed” water molecules, but these techniques



cannot capture the location of the majority of the waters in the active site (before or after the ligand binds).<sup>3,26,27</sup> In addition, a water molecule that diffracts simply implies that it is spatially immobilized but does not provide information about the energetics of the water.

To understand the thermodynamics of the network of waters in the active site of each HCA-ligand complex better we performed WaterMap calculations (see **Figure 4**),<sup>9,28,29</sup> which are explicit solvent calculations that determine a free energy, enthalpy, and entropy value for each molecule of water in the active site of the protein-ligand complex<sup>2,4</sup> using an approach called inhomogeneous solvation theory.<sup>30,31</sup> These calculations support the *H/S* compensation we observe in the ITC measurements of HCA-ligand binding, and show: i) **4-F<sub>1</sub>BTA** and **4,6-F<sub>2</sub>BTA** have the most favorable entropy of binding, and the least favorable enthalpy of binding. These effects result from subtle disruptions in the network of waters in the solvent-exposed region of the binding site, as seen in Figure 5 (the **4-F<sub>1</sub>BTA** results look highly similar to **4,6-F<sub>2</sub>BTA** and are therefore omitted for clarity). The fluorination pattern of these ligands apparently disrupts the water network in a way that results in less restricted water motion and therefore better entropy. ii) WaterMap calculates **5,6-F<sub>2</sub>BTA** to have the least favorable entropy of binding, a result that is also consistent with the experiment. The 5- and 6-positions of the benzo – extension are directed toward bulk solvent, and the difluoro substitution is predicted to restrict the mobility of a local region of waters around the ligand. Figure 5 shows an additional hydration site localized above the **5,6-F<sub>2</sub>BTA** ligand (denoted by a black arrow). WaterMap predicts an additional region of entropically unfavorable water to the side of the **5,6-F<sub>2</sub>BTA** ligand (denoted by the dashed oval), further contributing to the unfavorable entropy of binding. iii) **6,7-F<sub>2</sub>BTA** has a value for the entropy of binding between the values of **4,6-F<sub>2</sub>BTA** and **5,6-F<sub>2</sub>BTA**. As seen in Figure 5, the region to the right of the ligand (see dashed oval) is similarly unfavorable to **5,6-**

**F<sub>2</sub>BTA**, but the region extending toward bulk solvent does not have the additional localized hydration site observed in **5,6-F<sub>2</sub>BTA** (see hydration site indicated by the black arrow in **5,6-F<sub>2</sub>BTA** that is missing from **6,7-F<sub>2</sub>BTA**)

*The improved free energy of binding of **6,7-F<sub>2</sub>BTA** (compared to **H<sub>4</sub>BTA**) arises from desolvation of the ligand upon binding, and most likely is not from a rearrangement of the solvent in the active site of HCA. **6,7-F<sub>2</sub>BTA** is the only ligand that binds to HCA with a higher binding affinity than **H<sub>4</sub>BTA**; this difference in free energy of binding ( $\Delta G^{\circ}_{\text{bind, x-F}_y\text{BTA}} - \Delta G^{\circ}_{\text{bind, H}_4\text{BTA}} = \Delta \Delta G^{\circ}_{\text{bind, Fluorination}}$ ) is  $1.1 \pm 0.3 \text{ kcal mol}^{-1}$  more favorable (Figure S1). The increased binding affinity of **6,7-F<sub>2</sub>BTA** over **H<sub>4</sub>BTA** cannot be attributed to differences in buried hydrophobic area upon ligand binding (Figure S1), but arises from a more favorable entropy of binding. There are two plausible explanations for this increased binding affinity: i) the reorganization of the waters in the active site of HCA upon binding of the ligand are responsible for the more favorable  $-T\Delta S^{\circ}_{\text{bind}}$  of **6,7-F<sub>2</sub>BTA**; or ii) the desolvation of **6,7-F<sub>2</sub>BTA**, in addition to the desolvation of the active site of HCA, influences the  $\Delta G^{\circ}_{\text{bind}}$ .*

Entropy-driven binding is compatible with the mechanism of the hydrophobic effect proposed by Kauzmann and Tanford, but is the only thiazole-based ligand studied thus far that is not enthalpy-dominated. The contribution of the enthalpy ( $\Delta H^{\circ}_{\text{OW}}$ ) and entropy ( $-T\Delta S^{\circ}_{\text{OW}}$ ) of partitioning of **H<sub>4</sub>BTA** and **6,7-F<sub>2</sub>BTA** to the free energy of partitioning ( $\Delta G^{\circ}_{\text{OW}}$ ) mirrors the contributions of  $\Delta H^{\circ}_{\text{bind}}$  and  $-T\Delta S^{\circ}_{\text{bind}}$  in the binding of these ligands to HCA (Figure S1).

WaterMap predicts **6,7-F<sub>2</sub>BTA** to be roughly equientropic with **H<sub>4</sub>BTA**. WaterMap only accounts for first-order water correlation terms and only considers regions of high water density for the thermodynamic calculations and not for desolvation of the ligand. The most plausible explanation for this mirroring of trends of partitioning and binding is therefore that the

desolvation of the ligand, and not just the desolvation of the active site of HCA, influences the  $\Delta G^{\circ}_{\text{bind}}$ .

*The HS compensation observed in the binding of **4-F<sub>1</sub>BTA** and **4,6-F<sub>2</sub>BTA** arise from a reorganization of the solvent.* The values of  $\Delta H^{\circ}_{\text{bind}}$  and  $-\text{T}\Delta S^{\circ}_{\text{bind}}$  of **4-F<sub>1</sub>BTA** and **4,6-F<sub>2</sub>BTA** are significantly different than **H<sub>4</sub>BTA** ( $> 4 \text{ kcal mol}^{-1}$ ), but compensate and result in unchanged binding affinities. Interestingly, these ligands are the only two that rotate within the active site (Figure 2), possibly to reduce the unfavorable interaction of the fluorine at the 4-position with the backbone carbonyl of Thr 200 if the pose did not undergo rotation. The conserved binding of these ligands to HCA, and the conserved structure of the side chains of the amino acids of HCA in the active site suggest that neither changes in structure nor interaction of the protein and ligand are plausible candidates for significant changes in the thermodynamics binding. The most plausible candidate for the source of these compensated changes in  $\Delta H^{\circ}_{\text{bind}}$  and  $-\text{T}\Delta S^{\circ}_{\text{bind}}$  is therefore the networks of hydrogen bonds of waters in the active site, and surrounding the ligands in solution and in the protein-ligand complex. WaterMap predicts the same trends for the observed thermodynamic of binding; the changed networks of water of **4-F<sub>1</sub>BTA** and **4,6-F<sub>2</sub>BTA** in the active site of HCA clearly indicate the importance of water in *H/S* compensation (Figure 5).

## Conclusions and interpretations

The results obtained from the thermodynamic analysis, X-ray crystal structures, and molecular dynamics simulations described in this work show that a series of ligands with different electronic structure bind to HCA with very similar values of  $\Delta G^{\circ}_{\text{bind}}$ , but with very different (and compensating) values of  $\Delta H^{\circ}_{\text{bind}}$  and  $-T\Delta S^{\circ}_{\text{bind}}$ . These results suggest that the “size” (a term we cannot presently disaggregate into surface area, molecular volume, or dipole moment) of the ligand, and thus the water that is displaced from, or perturbed in the active site of HCA, is primarily responsible for the  $\Delta G^{\circ}_{\text{bind}}$ ; it also implies that changes in the structure of the networks of hydrogen bonded waters, that result upon ligand binding, determine the values of  $\Delta H^{\circ}_{\text{bind}}$  and  $-T\Delta S^{\circ}_{\text{bind}}$ . This water-centric view of ligand binding—and *H/S*-compensation—cannot be rationalized by the lock-and-key principle, and suggest that the molecules of water surrounding the ligand and filling the active site of a protein are as important as the structure of the ligand and the surface of the active site.

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## **Author Contributions**

B.B., M.R.L. and G.M.W. designed research; B.B., M.R.L, W.S., S.F., M.A. H.L, C.M.B., and A.H. performed research; B.B., M.R.L, W.S., S.F., M.A. H.L, C.M.B., and A.H. analyzed data; and B.B, M.R.L., W.S., and G.M.W. wrote the paper.

## **Competing financial interests**

The authors declare no competing financial interests.

## **Additional Information**

Supplementary information is available online at <http://www.nature.com/naturechemicalbiology>. Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>. Supplementary information details the experimental procedures for the synthesis of the ligands, the purification of protein, the measurement of the thermodynamics of binding and partitioning, the measurement of the pK<sub>a</sub> of the ligands, the preparation and crystallography of the protein–ligand complexes, and the molecular dynamics calculation for the HCA–ligand complexes. Correspondence and requests for materials should be addressed to G.M.W.

## Figure Legends

**Figure 1:** (a) Structures of the partially fluorinated ligands used in this study, and their abbreviations. The abbreviation of each ligand indicates the number of fluorine atoms on the benzo-extension (e.g., **F<sub>2</sub>BTA** contains two fluorine atoms). (b) An overlay of the heavy atoms of the **H<sub>4</sub>BTA** and **F<sub>4</sub>BTA** ligand from (aligned) crystal structures of the two HCA-ligand complexes. (c) Diagram of the amino acid residues of HCA that form contacts with the benzothiazole (**H<sub>4</sub>BTA**) and per-fluorobenzothiazole (**F<sub>4</sub>BTA**) sulfonamide ligands, determined previously from crystal structures of each complex. Favorable interactions between the ligand and the protein are represented with a blue dashed line, and unfavorable interactions with a red dashed line.

**Figure 2.** Crystal structures of the active site of HCA complexed with: (a) **4-F<sub>1</sub>BTA**, (b) **7-F<sub>1</sub>BTA**, (c) **4,6-F<sub>2</sub>BTA**, (d) **6,7-F<sub>2</sub>BTA**, (e) **4,7-F<sub>2</sub>BTA**, and (f) **5,6-F<sub>2</sub>BTA**. To the right of each crystal structure is an overlay of the heavy atoms of the ligand with **H<sub>4</sub>BTA** or **F<sub>4</sub>BTA** from aligned structures of the two HCA-ligand complexes: (a) **4-F<sub>1</sub>BTA** and **F<sub>4</sub>BTA**; (b) **7-F<sub>1</sub>BTA** and **H<sub>4</sub>BTA** (c) **4,6-F<sub>2</sub>BTA** and **F<sub>4</sub>BTA**; (d) **6,7-F<sub>2</sub>BTA** and **H<sub>4</sub>BTA**; (e) **4,7-F<sub>2</sub>BTA** and **F<sub>4</sub>BTA**; and (f) **5,6-F<sub>2</sub>BTA** and **H<sub>4</sub>BTA**

**Figure 3** Diagram of the  $pK_a$ -corrected thermodynamic results  $\Delta\Delta J_{\text{bind}}^0$  (compared to **H<sub>4</sub>BTA**)—where  $J = G$  (blue),  $H$  (green), and  $S$  (red)—obtained from ITC measurements. The relative differences in the enthalpy and entropy of binding (i.e., mutual  $H/S$  compensation) result in indistinguishable values of  $\Delta G_{\text{bind}}^0$  for **4-F<sub>1</sub>BTA**, **5,6-F<sub>2</sub>BTA**, **4,7-F<sub>2</sub>BTA**, **4,6-F<sub>2</sub>BTA**, **4,5,6-**

**F<sub>3</sub>BTA**, and **F<sub>4</sub>BTA**. The gray region demarcates the 95% confidence interval (i.e., two standard deviations) of  $\Delta\Delta G_{\text{bind}}^{\circ}$  for **H<sub>4</sub>BTA**.

**Figure 4.** Diagram comparing of the  $pK_{\text{a}}$ -corrected thermodynamics of  $\Delta\mathcal{S}_{\text{bind}}^{\circ}$  results from ITC measurements with WaterMap calculations for  $\Delta G_{\text{bind}}^{\circ}$  (a),  $\Delta H_{\text{bind}}$  (b), and  $-T\Delta S_{\text{bind}}^{\circ}$  (v).

**Figure 5.** Hydration site thermodynamics for **H<sub>4</sub>BTA** and three fluorinated variants (**4,6-F<sub>2</sub>BTA**, **5,6-F<sub>2</sub>BTA**, and **6,7-F<sub>2</sub>BTA**). Hydration site colors range from green (favorable) to red (unfavorable). The range for  $\Delta H_{\text{bind}}^{\circ}$  (left panel) is  $[-5.0 \text{ to } +5.0 \text{ kcal mol}^{-1}]$  whereas for  $-T\Delta S_{\text{bind}}^{\circ}$  (right panel) the range is  $[0.0 \text{ to } +5.0 \text{ kcal mol}^{-1}]$ . WaterMap computes all values relative to bulk solvent. The black arrow indicates an additional localized hydration site in **5,6-F<sub>2</sub>BTA** that is not observed in other **x-F<sub>2</sub>BTA** variants. The dashed oval indicates a cluster of hydration sites that is entropically unfavorable in **5,6-F<sub>2</sub>BTA** and **6,7-F<sub>2</sub>BTA** relative to **4,6-F<sub>2</sub>BTA**.

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